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Direct Assessment of MHC Class I Binding by Seven Ly49 Inhibitory NK Cell Receptors

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Summary

Mouse NK cells express at least seven inhibitory Ly49 receptors. Here we employ a semiquantitative cell–cell adhesion assay as well as class I/peptide tetramers to provide a comprehensive analysis of specificities of Ly49 receptors for class I MHC molecules in eight MHC haplotypes. Different Ly49 receptors exhibited diverse binding properties. The degree of class I binding was related to the extent of functional inhibition. The tetramer studies demonstrated that neither glycosylation nor coreceptors were necessary for class I binding to Ly49 receptors and uncovered peptide-specific recognition by a Ly49 receptor. The results provide a foundation for interpreting and integrating many existing functional studies as well as for designing tests of NK cell development and self-tolerance.

Introduction

Recognition of MHC class I molecules on target cells is a key event in the regulation of NK cell activity. Class I–specific inhibitory receptors expressed on the surface of NK cells prevent them from lysing target cells expressing a cognate class I ligand. Consequently, cells with extinguished class I expression often become target cells for NK cells (Ljunggren and Karre, 1990).

In the mouse, the only known NK cell receptors for classical class I molecules belong to the Ly49 family (Yokoyama, 1995). Seven well described Ly49 family members containing inhibitory motifs in their cytoplasmic domain (Ly49A, -B, -C, -E, -F,- G2, and -I) are known to be expressed at least at the mRNA level by NK cells from C57BL/6 (B6) mice. Based on functional studies, Ly49A was initially attributed a specificity for D^d but not K^d, L^d, K^b, or D^b (Karlhofer et al., 1992). Direct binding of Ly49A to D^d was demonstrated in a cell–cell adhesion assay (Daniels et al., 1994a; Takei et al., 1997) or by binding of Ly49A-expressing cells to soluble, plate-bound D^d molecules (Kane, 1994). Ly49A also binds to H-2^k cells (Brennan et al., 1996b). Although genetic evidence has implicated D^k as the ligand (Karlhofer et al., 1994), anti-D^k antibodies reportedly failed to block adhesion of Ly49A-transfected cells to H-2^k cells (Brennan et al., 1996b).

Because Ly49G2$^+$ NK cells lysed H-2^d cells more efficiently in the presence of mAbs against D^d or L^d, this receptor was assigned a specificity for D^d and L^d (Mason et al., 1995). In vivo, Ly49G2-expressing NK cells were capable of rejecting bone marrow allografts from H-2^b, but not H-2^d donors, consistent with the notion that Ly49G2 is an inhibitory receptor for H-2^d (Raziuddin et al., 1996). A recent report implicates D^d but not L^d as a ligand for Ly49G2-expressing NK cells in vivo (Johansson et al., 1998). However, direct evidence for a physical interaction between Ly49G2 and D^d has not been reported.

The MHC specificities of the closely related Ly49C and Ly49I receptors (Brennan et al., 1996a) were initially studied in bone marrow graft rejection experiments. It was concluded that Ly49C and/or Ly49I recognize the K^b molecule (Yu et al., 1996). Cell adhesion experiments with Ly49C- and Ly49I-transfected COS-7 cells and MHC disparate tumor cell lines have demonstrated that Ly49C binds to H-2^b, H-2^d, H-2^k, and H-2^s gene products, while Ly49I was not found to bind any of these (Brennan et al., 1996a, 1996b). To date, little is known about the expression and MHC reactivity of any of the other Ly49 receptors with predicted inhibitory function (Ly49B, -E, and -F). Ly49D exhibits activating rather than inhibitory function in response to target cells expressing the D^d class I molecule (George et al., 1999; Nakamura et al., 1999), but no binding data has been reported.

Evidence indicates that Ly49A interacts with the α2 domain of D^d (Daniels et al., 1994a; Sundbäck et al., 1998), with possible contributions from the α1 domain (Matsumoto et al., 1998). The functional binding of Ly49A to D^d showed no peptide specificity, since a variety of peptides that stabilize D^d expression on target cells protect these from lysis by Ly49A$^+$ NK cells (Correa and Raulet, 1995; Orihuela et al., 1996). The role of D^d glycosylation has been investigated based upon the lectin-like structure of Ly49 receptors and the capacity of both Ly49A and Ly49C to bind the sulfated glycan fucoidan

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(Daniels et al., 1994b; Brennan et al., 1995). Class I molecules on target cells treated with glycosylation inhibitors or glycosidases reportedly failed to inhibit NK cells (Daniels et al., 1994b; Brennan et al., 1995). However, mutation of the N-glycosylation acceptor sites in D^d did not reduce inhibitory NK cell recognition in one study (Matsumoto et al., 1998) and reduced it only partially in another (Lian et al., 1998). Thus, the role of class I–linked glycans in Ly49 interactions is not fully resolved.

Ly49 receptors are expressed on partially overlapping subpopulations of NK cells (reviewed in Raulet et al., 1997). The missing self hypothesis proposes that NK cells should be inhibited by the few self–class I molecules inherited by each individual. It is believed that "education" processes ensure that many or all functional NK cells express self–class I specific receptors (Hoglund et al., 1997; Raulet et al., 1997). A direct assessment of whether each NK cell expresses at least one self–class I–specific receptor will require knowledge of which Ly49 receptors interact with which MHC ligands. Such information may also aid in elucidating the features of class I molecules that are recognized by Ly49 receptors. To date, the specificities of only a few Ly49 receptors have been examined and only for a few MHC haplotypes. Here, we employ two binding assays and a functional assay to examine the specificities of the panel of inhibitory Ly49 receptors for eight MHC haplotypes.

Results

MHC Allele Specificities of Inhibitory Ly49 Molecules

We initially employed a semiquantitative cell adhesion assay to investigate Ly49 specificity. Ly49-transfected COS-7 cells were adhered to ^{3}H-labeled concanavalin A (Con A) blasts from MHC congenic, MHC recombinant, and MHC mutant mice. The use of MHC congenic Con A blasts instead of tumor cells as in earlier studies circumvented the problem of non-MHC variability in adhesive properties among unrelated tumor cell lines.

All Ly49 members were expressed at high levels on the surface of transfected COS-7 cells. The equivalently strong staining intensity of Ly49C, Ly49F, and Ly49I transfectants with the Ly49C, -F, -I, and -H reactive mAb 14B11 (Corral et al., 1999) indicates that these molecules were expressed at comparable levels (Figure 1A). Ly49C and Ly49I transfectants also stained equivalently with the Ly49C- and Ly49I-reactive mAb 5E6 (data not shown). Similarly, Ly49A transfectants stained strongly with mAb JR9–318, Ly49G2 transfectants with mAb 4D11, and Ly49D transfectants with mAb 4E5 (Figure 1A). Since no Ly49B- or Ly49E-reactive mAbs are available, hemagglutinin (HA) epitope tags were added to the C termini of these molecules, and surface expression was monitored with anti-HA mAb. Control experiments with Ly49A showed that the HA tags did not affect the extent of binding in the cell–cell adhesion assay. Ly49B and Ly49E were expressed to a similar degree as the control-tagged Ly49A (Figure 1B).

We readily observed specific binding of most Ly49 transfectants to Con A blasts from one or more of the B10 MHC congenic mice tested (Figure 2). In cases where Ly49 transfectants bound to Con A blasts from H-$2^{b,\ d,\ or\ k}$ mice, no binding was observed with class

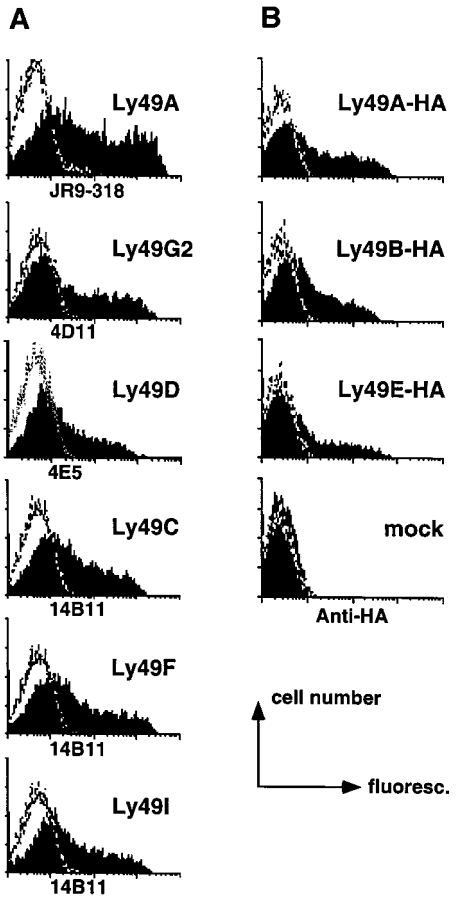

Figure 1. Cell Surface Expression of Ly49 Receptors on COS-7 Transfectants

(A) COS-7 cells were transiently transfected with the indicated Ly49 cDNAs (filled curve) or a negative control (inverted Ly49G2 cDNA, open curve). After 2 days, cells were stained with the indicated FITC-conjugated mAbs.

(B) COS-7 cells transfected with HA-tagged Ly49A, Ly49B, Ly49E, or control cDNA were stained with anti-HA (filled curve) or an isotype control mAb (open curve) and donkey anti-MIg-PE.

I–deficient blasts from comparison strains that were homozygous for a mutation in the β*2 microglobulin* (β*2m*) gene (H-$2^{b,\ d,\ or\ k}$) or the *TAP-1* gene (H-2^b) (Figure 2; Table 1; data not shown). In contrast, deficiency of class II molecules had no effect in the assay (in the case of H-2^b [data not shown]).

In accordance with previous data (Daniels et al., 1994a; Brennan et al., 1996b), Ly49A transfectants bound strongly to Con A blasts of the H-2^d and H-2^k but not the H-2^b haplotype. In eight independent experiments, binding of Ly49A to H-2^d was consistently higher (about 1.8-fold) than binding to H-2^k (Figure 2; data not shown). In contrast to a previous report (Brennan et al., 1996b), Ly49A transfectants bound significantly to H-2^s cells, albeit weakly. Interestingly, Ly49A transfectants also bound Con A blasts of all other haplotypes tested, i.e., H-$2^{f,q,r,v}$. The results indicate that Ly49A exhibits a broad and often strong MHC reactivity.

Consistent with functional evidence that Ly49G2 interacts with D^d and/or L^d (Mason et al., 1995), Ly49G2-transfected cells reproducibly bound to H-2^d cells. This

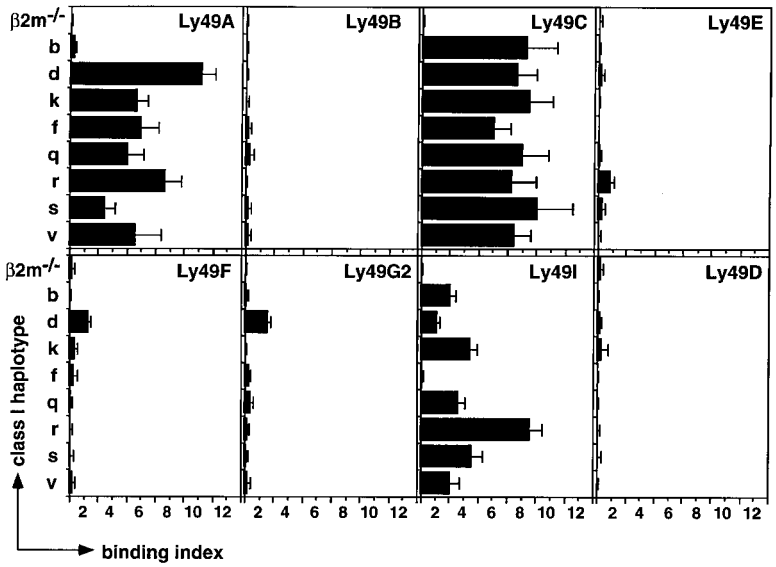

Figure 2. Cell–Cell Adhesion Assay Detects Binding of Ly49 Receptors to Lymphoblasts from MHC Congenic Mice

Ly49-transfected COS-7 cells were adhered to ^{3}H-labeled Con A blasts from B6 $\beta 2m^{-/-}$ ($\beta 2m^{-/-}$), B10 (b), B10.D2 (d), B10.BR (k), B10.M (f), B10.Q (q), B10.RIII (r), B10.S (s), and B10.SM (v) mice (MHC haplotype in parentheses). The binding index was determined as "cpm Ly49 transfectants / cpm mock transfectants." Means ± SEM of three to ten independent experiments are shown.

interaction was not detected in a previous binding study (Takei et al., 1997). Binding by Ly49G2 was substantially lower than binding by Ly49A-transfected cells in each of eight independent comparisons. Interestingly, Ly49G2 displayed little if any reactivity toward any of the other seven MHC haplotypes tested, suggesting that Ly49G2 is highly selective for MHC binding.

Ly49C reacted strongly with Con A blasts of all eight MHC congenic strains tested. These data are consistent with previous results showing that Ly49C interacts with K^b and H-$2^{d,k,s}$ molecules expressed on tumor cells (Brennan et al., 1996b) and extend the MHC specificity of Ly49C to new class I alleles. The closely related Ly49I molecule reacted with H-2^r cells to a similar extent as Ly49C. However, Ly49I bound all other MHC haplotypes more weakly than did Ly49C and failed to bind H-2^f cells at all. A previous study failed to detect Ly49I binding to MHC molecules (Takei et al., 1997).

Ly49F, also similar in sequence to Ly49C and Ly49I, bound weakly but consistently to MHC molecules of the H-2^d haplotype but failed to bind cells of all the other haplotypes. Ly49B, Ly49D, and Ly49E displayed little, if any, MHC reactivity to any haplotype in the adhesion assay.

In each case where the binding of Ly49-transfected cells to H-2^d lymphoblasts occurred, the binding could be blocked by corresponding anti-Ly49 mAbs. Thus, binding by Ly49A-transfectants was blocked by mAb A1, binding by Ly49G2 transfectants was blocked by mAb 4D11, and binding by Ly49C, -F, and -I transfectants was blocked by mAb 14B11 (Table 1). Isotype control mAbs had no effect (data not shown).

MHC Locus Specificities of Inhibitory Ly49 Molecules

For more detailed analysis of MHC specificities, Con A blasts from MHC recombinant mice were used in the

Table 1. Class I Specificity of Ly49 Binding in Cell–Cell Adhesion Assay

Adhesion Partners	H-2 KDL[1]	mAb	Transfectants Ly49A	Ly49C	Ly49F	Ly49G2	Ly49I
B6/B10	bb−	-	1.0 ± 0.1	8.0 ± 4.7	1.1 ± 0.1	1.0 ± 0.1	2.3 ± 0.5
B6 $\beta 2m^{-/-}$		-	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
B10.D2	ddd	-	7.3 ± 1.4	6.9 ± 1.7	2.2 ± 0.4	2.7 ± 0.2	1.9 ± 0.4
B6-H-2^d-$\beta 2m^{-/-}$		-	1.0 ± 0.1	1.0 ± 0.2	1.6 ± 1.0	1.2 ± 0.1	1.1 ± 0.2
B10.BR	kk−	-	4.2 ± 1.4	8.5 ± 3.5	1.5 ± 0.3	1.0 ± 0.1	3.2 ± 1.1
B10.BR $\beta 2m^{-/-}$		-	0.9 ± 0.2	1.0 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
B10.HTG	db−	-	1.5 ± 0.1	3.6 ± 1.4	2.5 ± 0.8	1.3 ± 0.1	2.9 ± 1.0
B10.D2(R107)	bdd	-	9.3 ± 2.3	7.7 ± 2.1	1.0 ± 0.1	3.1 ± 0.5	4.0 ± 1.5
B10.A(2R)	kb−	-	1.0 ± 0.1	7.2 ± 2.7	1.3 ± 0.2	1.1 ± 0.1	1.5 ± 0.1
B10.D2	ddd	Ly49[2]	1.1 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	1.3 ± 0.2	0.9 ± 0.1
B10.D2	ddd	D^d [3]	1.2 ± 0.4	5.4 ± 2.0	1.1 ± 0.4	0.8 ± 0.0	0.9 ± 0.1
B10.BR	kk−	D^k,K^k [4]	1.1 ± 0.2	1.2 ± 0.2	0.8 ± 0.3	0.7 ± 0.1	0.6 ± 0.2
B6/B10	bb−	K^b [5]	nd	0.8 ± 0.2	0.9 ± 0.1	nd	0.8 ± 0.1

Data represents mean binding index ± SEM of three to six independent semiquantitative adhesion experiments with the indicated COS-7 transfectants and Con A blasts from the listed strains. The index was calculated in reference to COS-7 cells transfected with an inverted Ly49G2 cDNA.

[1] The H-2 designations refer to allelic variants at K, D, and L. The hyphen means that no L product is made in this strain.

[2] Anti-Ly49 mAbs used for blocking (50 μg/ml) were A1 (anti-Ly49A), 14B11 (anti-Ly49C,F,I), and 4D11 (anti-Ly49G2). Anti–class I mAbs were 34-5-8S[3] (anti-D^d $\alpha 1/\alpha 2$), 15-1-5P[4] (anti-K^k, D^k), and Y3[5] (anti-K^b). nd, not determined.

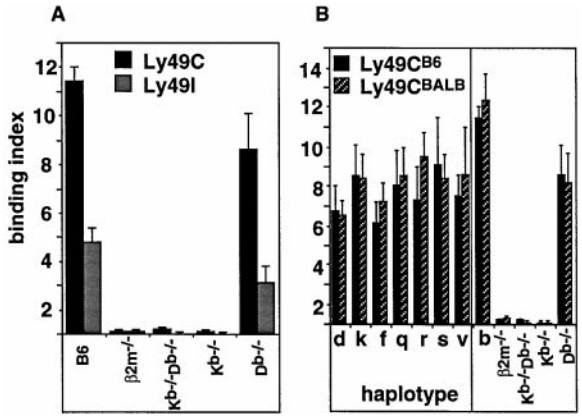

Figure 3. MHC Specificities of Ly49I, Ly49C^{B6}, and Ly49C^{BALB}

(A) Ly49C and Ly49I bind to K^b molecules on lymphoblasts but not appreciably to D^b molecules. The adhesion assay was performed with Ly49C or Ly49I transfectants and Con A blasts from B6 mice or B6 mice deficient for β2m, D^b, K^b, or both K^b and D^b. Means ± SEM of four to six determinations from two to four independent experiments are shown.

(B) Ly46C^{B6} and Ly49C^{BALB} are indistinguishable in MHC binding. The adhesion assay was performed as described in Figure 2. Means ± SEM of three to six independent experiments are shown.

adhesion assay, and anti-class I mAbs were employed to block binding (Table 1). Confirming the specificity of Ly49A for D^d was the finding that anti-D^d mAb blocked binding of Ly49A transfectants to B10.D2 cells (Table 1). Furthermore, Ly49A-transfected cells bound to B10.D2(R107) cells, which express K^b, D^d, and L^d, and did not bind to B10.HTG cells, which express K^d but are otherwise H-2^b (Table 1). Binding of Ly49A transfectants to H-2^k cells was blocked with a mAb that reacts with both K^k and D^k. Significantly, the Ly49A transfectants did not bind to B10.A(2R) cells (K^k/D^b), consistent with previous genetic results indicating that Ly49A interacts with D^k and not K^k (Karlhofer et al., 1994).

As was the case for Ly49A, Ly49G2 transfectants bound to B10.D2(R107) cells but not to B10.HTG cells, implicating D^d and/or L^d in binding. Binding to H-2^d cells was blocked to background levels by mAb specific for D^{d}α1/α2 (Table 1). These data confirm that D^d is a ligand for Ly49G2, in contrast to a report that questioned this assignment (Takei et al., 1997). The complete blockade with anti-D^d mAb suggests that L^d is not an effective ligand for Ly49G2, in contrast to a previous study suggesting that both molecules are ligands (Mason et al., 1995). Since Ly49G2 bound significantly only to D^d among eight haplotypes tested, this receptor exhibits a very high degree of selectivity in MHC binding.

The binding of Ly49C and Ly49I transfectants to H-2^b Con A blasts was completely blocked with a specific anti-K^b mAb, confirming that K^b is essential for both of these interactions (Table 1; Brennan et al., 1996b). This conclusion was strongly supported by analysis of binding of Con A blasts from mice with targeted mutations in *K^b*, *D^b*, or both (Perarnau et al., 1999). Cells with the *K^b* mutation failed to bind to either Ly49C- or Ly49I-transfected cells (Figure 3A). In contrast, cells with the *D^b* mutation bound nearly as well as wild-type cells. Therefore, K^b is the major H-2^b class I ligand for Ly49C and Ly49I and D^b plays no role or a very minor one.

Binding of Ly49C or Ly49I to H-2^k cells was completely blocked by an antibody that reacts with both K^k and D^k, demonstrating interactions with one or both of these class Ia molecules. Binding of Ly49C to H-2^d blasts was only partially blocked by anti-D^d mAb, consistent with the previous report that K^d may also bind Ly49C (Brennan et al., 1996b). Binding of Ly49I to H-2^d molecules was reduced to background with anti-D^d mAb, arguing that D^d is the major H-2^d ligand for Ly49I. However, since Ly49I also bound significantly to B10.HTG blasts (K^{d}D^b), K^d and/or D^b could not be ruled out as ligands for Ly49I. H-2^d was the only haplotype displaying reactivity with Ly49F above background. Since anti-D^d mAb blocked binding to B10.D2 cells, but Ly49F also reacted with B10.HTG (K^{d}D^b) cells, the locus specificity of Ly49F remains uncertain.

Different Ly49C Alleles Bind the Same Class I Molecules

The Ly49C protein exhibits allelic diversity, differing in four amino acids between the B6 and BALB strains (Brennan et al., 1996a). We compared binding of Ly46C^{B6} and Ly49C^{BALB} transfectants in the adhesion assay. As shown in Figure 3B, we observed no difference in class I reactivity between Ly46C^{B6} and Ly49C^{BALB}. Supporting this finding, Ly46C^{B6} and Ly49C^{BALB} transfectants were also bound equally well by soluble MHC class I/peptide tetramers of the H-2^{b,d,k} haplotypes (data not shown). Therefore, we find no evidence for differences in the specificity of these allelic Ly49C receptors.

Avidity for Class I Determines the Inhibitory Function of Ly49A and Ly49G2

To address whether the differences in class I binding by Ly49 molecules as detected in our assay is related to the degree of inhibitory function, we correlated binding in the cell–cell adhesion assay with the capacity of MHC-different spleen cells to inhibit the function of Ly49-expressing lymphocytes. For these experiments, we took advantage of the fact that transgenically expressed Ly49 molecules on T cells readily inhibit the proliferative response of the T cells to allogeneic stimulator cells expressing cognate class I ligands (Held et al., 1996a). The use of T cells also has the important advantage that very few of these cells express additional endogenously encoded Ly49 receptors that might confound the specificity analysis. We therefore employed Ly49A transgenic mice (Held et al., 1996a) and a new Ly49G2 transgenic line as a source of T cells to examine MHC specificity. Similar to the Ly49A transgene, the Ly49G2 transgene was expressed on all T cells at homogeneously high levels (Figure 4A). T cells from transgenic or nontransgenic littermates on the B6 (H-2^b) background were used as responder cells with spleen cells from MHC congenic mice as allogeneic stimulator cells.

The relative inhibition of T cell proliferation by the Ly49A transgene corresponded quite well with the class I avidity of Ly49A as determined by the adhesion assay (Figure 4). Inhibition was most prominent with H-2^d stimulator cells and least prominent with H-2^s stimulator cells. The other haplotypes showed intermediate inhibition effects that correlated with their binding properties, except in the case of the H-2^f stimulator cells, which

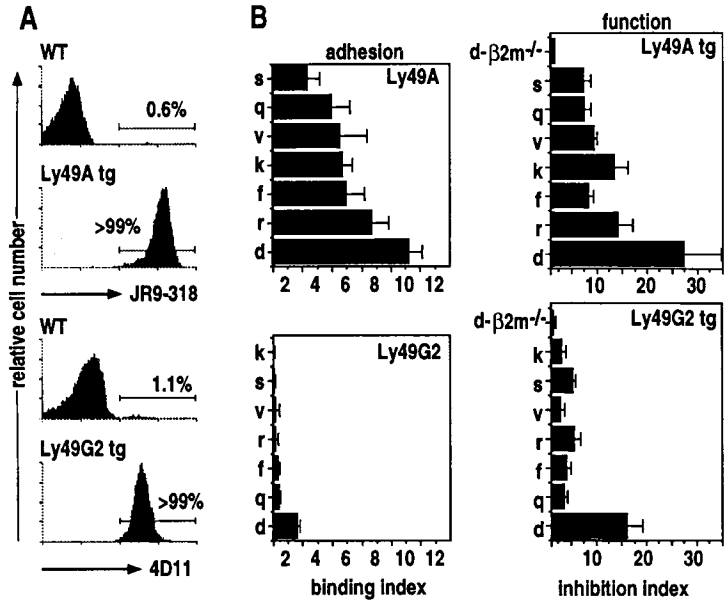

Figure 4. The Inhibitory Function of Ly49A and Ly49G2 Is Related to the Extent of Binding as Determined in the Cell–Cell Adhesion Assay

(A) Ly49A and Ly49G2 transgenic mice express the corresponding Ly49 receptor at high levels on T cells. JR9–318 (upper panel) or 4D11 (lower panel) staining of gated $CD3^+$ nylon wool–passed lymph node cells is shown for Ly49A transgenic mice (Ly49A tg), Ly49G2 transgenic mice (Ly49G2 tg), or nontransgenic littermates (wt).

(B) Relationship of class I binding and inhibitory function. The T cells represented in (A) were used as responder cells in mixed lymphocyte reactions with irradiated spleen cells from the indicated MHC congenic strains as stimulator cells. Relative binding data are taken from Figure 2. The inhibition index was determined as the stimulation index with nontransgenic responder cells / the stimulation index with transgenic responder cells. The data represent means ± SEM of four independent experiments, each determined in triplicate, except in the case of the H-2^d $\beta 2m^{-/-}$ stimulator cells, where the data represent the means of two (Ly49A tg) or three (Ly49G2 tg) experiments.

exhibited slightly less than expected levels of inhibition. For Ly49G2 transgenic responder cells, the T cell response was most strongly inhibited by H-2^d stimulator cells, in agreement with D^d being the dominant ligand for Ly49G2 in the adhesion assay. The inhibition was relatively strong in comparison to the relatively weak binding of Ly49G2 to H-2^d cells in the adhesion assay (see Discussion). The other haplotypes exhibited only weak inhibitory function. The extent of inhibition observed with H-2^d-$\beta 2m^{-/-}$ stimulator cells, which present foreign MHC class II molecules but no potentially inhibitory class I molecules, was minimal in the case of both Ly49A and Ly49G2 transgenic responder cells. Thus, the receptors do not cause significant inhibition in the absence of class I ligands. Taken together, these results demonstrate a clear relationship between the class I molecules that bind in the cell–cell adhesion assay and those that cause inhibition. The results with Ly49A allow the additional conclusion that inhibitory signaling is not an all or none phenomenon, since even weakly binding MHC haplotypes (e.g., H-2^s) exhibited some inhibitory function. Therefore, the other instances of relatively weak binding of inhibitory Ly49 receptors to class I molecules are likely to have functional significance.

Binding of Ly49 Molecules to Soluble MHC Class I/Peptide Tetramers

To complement the cell–cell adhesion assay in addressing the MHC specificities of Ly49 receptors, we examined the binding of fluorochrome-labeled tetrameric class I/β2m/peptide complexes to Ly49-transfected cells. This assay represents a more direct measurement of binding to Ly49 receptors, free from the hypothetical influence of non-MHC molecules on class I–Ly49 interactions, and also allows an assessment of the role of glycans and MHC-bound peptides in Ly49 binding.

Ly49-transfected COS-7 cells were stained with K^b, D^b, K^d, D^d, L^d, and D^k tetramers and analyzed by flow cytometry (Figure 5A). All of the tetramers except L^d exhibited specific binding to transfected COS cells expressing at least one of the inhibitory Ly49 receptors. No binding of Ly49-transfected COS-7 cells to nonclassical Qa-1^b tetramers, the ligand for CD94/NKG2A (Vance et al., 1998), was observed (data not shown). These data demonstrate direct binding of class Ia molecules to Ly49 receptors. Furthermore, since the class I molecules (with the exception of D^d) were generated in bacteria and are not glycosylated, the results indicate that N-glycosylation of class I molecules is not required for Ly49 binding.

As expected, Ly49A transfectants stained specifically with D^d and D^k tetramers but not with the other six tetramers. The binding of D^k tetramers corroborates the genetic evidence for this interaction (Karlhofer et al., 1994). Ly49G2 transfectants displayed weak but selective and reproducible reactivity with D^d tetramers. Binding of the D^d tetramers to Ly49A or Ly49G2 was blocked by the anti-Ly49A mAb JR9-318 or the anti-Ly49G2 mAb 4D11, respectively (Figure 5B). The Ly49G2 transfectants failed to stain with the K^b, D^b, K^d, and D^k tetramers. No reactivity with L^d tetramers was observed in two experiments (e.g., Figure 5), but very weak reactivity was observed in two others (data not shown). The L^d tetramers effectively stained LCMV-immune T cells, demonstrating their functionality (data not shown). In line with the cell–cell adhesion results, these data imply that L^d does not bind appreciably to Ly49G2.

Interestingly, Ly49C reacted with all class I tetramers except L^d, corroborating the promiscuity of this receptor observed in the cell–cell adhesion assay. Binding to K^b, K^d, D^d, and D^k is in accordance with the data from the adhesion assay and with results published previously (Brennan et al., 1996b). However, binding to D^b was

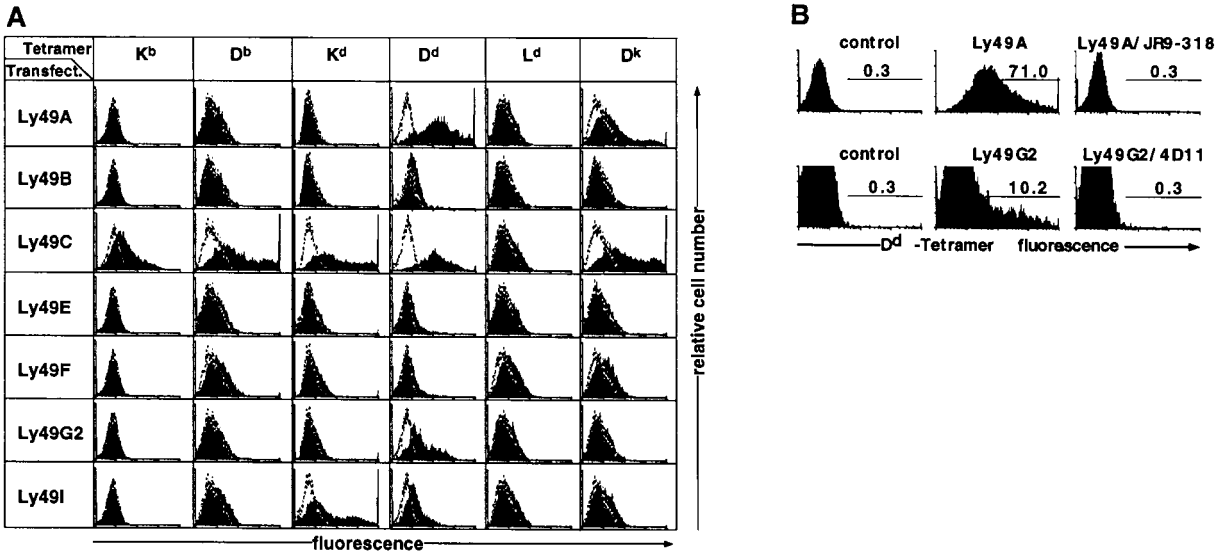

Figure 5. Distinct Ly49 Molecules Bind to MHC Class I Tetramers of the H-2^b, H-2^d, and H-2^k Haplotypes

(A) Ly49-transfected COS-7 cells were stained with MHC class I tetramers complexed to β2m and the following peptides: K^b, SEV9 (Sendai virus); D^b, gp33 (LCMV); K^d, JAK1 (self); D^d, gp49 (HIV); L^d, NP118 (LCMV); D^k, MT389 (polyoma virus). The filled curves represent Ly49 transfectants and the open curves control (inverted Ly49G2) transfectants. Quantitative comparisons are best made within a column, each of which represents an independent experiment. Ly49 expression on transfectants varied somewhat in different experiments. Histograms are representative of two to four experiments for each group.

(B) Anti-Ly49 mAbs inhibit staining with D^d tetramers. Ly49A-transfected or Ly49G2-transfected COS-7 cells were stained with D^d tetramers in the presence or absence of anti-Ly49A (JR9–318) or anti-Ly49G2 (4D11) mAbs, respectively. Staining of control Ly49G2^{inverse} transfectants is also shown.

surprising since Ly49C bound poorly, if at all, to D^b molecules expressed on Con A blasts in the cell–cell adhesion assay (Table 1; Figure 3) (Brennan et al., 1996b). The capacity of Ly49C transfectants to bind D^b tetramers was probably not due to unusual properties of the specific peptide bound to D^b, because equivalent binding was observed with D^b tetramer preparations containing two different bound peptides (LCMV NP396 and LCMV gp33 [data not shown]). D^b tetramers did not stain other Ly49 transfectants (Figure 5), and D^b tetramer binding to Ly49C was blocked by D^b-specific mAb B22–249 but not by K^b-specific mAb Y3 (data not shown). We conclude that Ly49C binds well to tetrameric recombinant D^b/peptide complexes but poorly, if at all, to D^b on lymphoblasts (see Discussion).

In two instances, interactions documented in the cell–cell adhesion assay were not detected in the tetramer studies. Ly49I-transfectants were stained well by K^d tetramers, weakly by D^d tetramers, and at background levels by K^b tetramers. The absence of reactivity with K^b tetramers contrasts with the unequivocal role of K^b in the adhesion assay, in which Ly49I transfectants failed to bind K^b knockout cells, and binding to wild-type cells was blocked with anti-K^b mAb (Table 1; Figure 3) (see Discussion). Ly49F, despite being a weak H-2^d-specific receptor in the adhesion assay (Figure 2; Table 1), was not detected with the K^d, D^d or L^d tetramers. In agreement with the adhesion experiments, Ly49B and Ly49E transfectants did not react significantly with any of the class I tetramers.

Ly49I Binding Depends on the Peptide Bound to K^d

We were able to examine the role of peptide in the binding of Ly49C and Ly49I to K^d with the use of a panel of K^d-peptide tetramers containing five unrelated peptides (Figure 6). Ly49C-transfected COS-7 cells bound specifically and equivalently to soluble K^d tetramers complexed to any of five different peptides. These data indicate that Ly49C exhibits little, if any, peptide specificity and further demonstrates the integrity of all

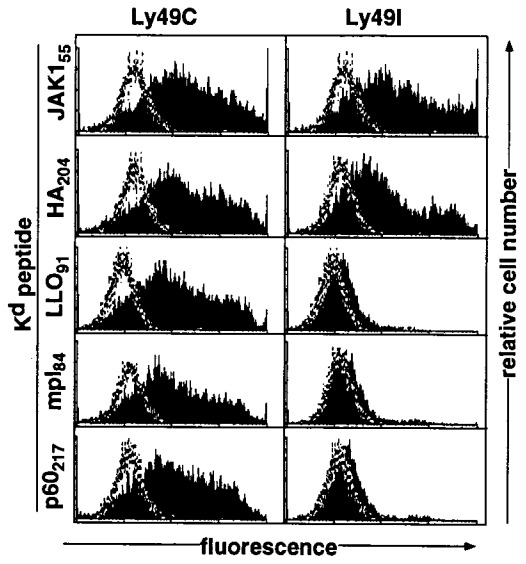

Figure 6. Ly49I but Not Ly49C Discriminates Peptides Bound to K^d

Ly49C, Ly49I (filled curves), or control (open curves) transfected COS-7 cells were stained with PE-conjugated K^d/β2m tetramers complexed to the indicated peptides. Identical results were obtained in a repeat experiment.

the tetramers. Interestingly, however, only two of the five peptides allowed binding of K^d to Ly49I. These data demonstrate that Ly49I can discriminate peptides presented by K^d.

Discussion

Evidence for a physical interaction with MHC class I molecules has previously only been reported for Ly49A with D^d and H-2^k and for Ly49C with K^b, K^d, D^d, and H-2^s gene products (Daniels et al., 1994a; Kane, 1994; Brennan et al., 1996b). MHC interactions with Ly49G2 and Ly49I have been inferred from functional studies (Mason et al., 1995; Yu et al., 1996) but have been questioned based on binding studies (Takei et al., 1997). Our cell–cell adhesion studies demonstrate several binding interactions that were not detected in previous binding studies, including Ly49G2 to H-2^d, Ly49A to H-2^s, and Ly49I to H-$2^{b,d,k,s}$. Most of these interactions were relatively weak, perhaps explaining the earlier failure to detect them. The Ly49G2–H-2^d interaction can be attributed mainly to the D^d class I molecule, since binding was blocked with anti-D^d mAb, and D^d but not L^d tetramers bound reproducibly to Ly49G2-transfected cells. The apparent lack of a role of L^d is at odds with some earlier functional results (Mason et al., 1995) but consistent with the results of a recent transgenic study implicating D^d but not L^d as a ligand inducing "missing self" reactivity in Ly49G2-expressing NK cells in vivo (Johansson et al., 1998). The cell–cell adhesion data also show that Ly49A and Ly49C bind strongly to a variety of additional MHC class I alleles, suggesting that common structural motifs define the class I specificities of these molecules (Figures 2–4; Table 1). In addition, evidence from the binding assay (Figure 3B) argues that allelic differences between Ly49C receptors do not contribute significantly to differences in class I specificity. More importantly, we report instances of MHC binding by Ly49I and Ly49F (Figures 2–4; Table 1).

It is interesting that some receptors exhibit such broad MHC cross-reactivity (e.g., Ly49C and Ly49A) while others are quite selective in MHC binding (e.g., Ly49G2). The more cross-reactive receptors provide an explanation for how such a small number of receptors can cover the much larger number of MHC alleles present in the population. The more selective receptors may be advantageous, since they would allow a host to detect the selective loss of particular MHC alleles. The receptors that failed to bind well to any of the tested MHC haplotypes, Ly49B and Ly49E, may have MHC ligands that were not represented in our panel. Ly49E in particular is very similar in sequence to other MHC-reactive Ly49 receptors (Ly49C, Ly49I, and Ly49F), suggesting a role in MHC recognition. Indeed, we observed very weak binding of Ly49E to H-2^r molecules in some experiments (Figure 2; data not shown). Ly49B, on the other hand, is unique in displaying by far the least homology to the other Ly49 family members (Wong et al., 1991), raising the possibility that it has a distinct function or specificity.

In the cases of Ly49A and Ly49G2, we provide evidence that the extent of binding observed in the cell–cell adhesion assay correlates with the extent of functional inhibition (Figure 4). By performing the assays with T cells, most of which do not express endogenously encoded Ly49 receptors (Ortaldo et al., 1998; our unpublished data), the contributions of other Ly49 receptors to inhibition is largely avoided. The most significant aspect of these data is that even weak binding by Ly49A can lead to inhibitory effects, suggesting that inhibition is not an all or none threshold phenomenon, but rather varies continuously with receptor avidity. The functional cross-reactivity of Ly49A with multiple MHC haplotypes was confirmed by limited analysis demonstrating that IL-2-activated NK cells expressing transgenic Ly49A are inhibited by Con A blasts from mice of several different MHC haplotypes (data not shown). The weak binding of Ly49G2 to D^d also resulted in significant functional inhibition in the T cell assay. The inhibition was relatively strong considering that binding to H-2^d cells by Ly49G2 was weaker than binding by Ly49A. This may occur because transgene-directed Ly49G2 levels were elevated on T cells compared to their normal expression level on NK cells (data not shown), or because Ly49G2 signaling is more efficient than Ly49A signaling. The finding that weak binding events lead to detectable inhibitory effects argues against the possibility that the other binding events we document are in some cases of too low an avidity to be functionally relevant. Equally pertinent, results from comparing NK cells that express Ly49A or Ly49G2 alone or together suggest that inhibitory effects mediated by two or more receptors on the same NK cell are cumulative (T. H. and D. H. R., unpublished data). Since NK cells commonly coexpress multiple Ly49 receptors, even the weak binding interactions we observe in the case of individual Ly49 receptors are likely to combine to produce a stronger inhibitory effect. Therefore, although we have not documented functional effects for all the binding events observed in the cell–cell adhesion assay, they probably play physiologically significant roles in the overall inhibitory interaction.

The binding data provide potential explanations for two previous observations. Of the seven well-characterized Ly49 receptors with ITIMs, only Ly49C and Ly49I bind significantly to H-2^b class I molecules. In contrast, five of the seven inhibitory Ly49 receptors react appreciably with H-2^d class I molecules. The large number of H-2^d-specific inhibitory receptors and the relative dearth of H-2^b-specific receptors may provide an explanation for the fact that NK cells in H-$2^{b/d}$ mice reject H-2^d bone marrow very inefficiently, while they reject H-2^b bone marrow grafts readily (Murphy et al., 1990). Furthermore, the abundance of H-2^d-specific receptors could account for the counterintuitive observation that the frequencies of NK cells that express Ly49A or Ly49G2 are marginally lower in H-2^d mice than in H-2^b mice (Held et al., 1996b), even though it might be expected that cells expressing H-2^d-specific (i.e., self-specific) receptors, as a group, would be more frequent in H-2^d mice. Mathematical modeling of proposed NK cell "education" processes led to the prediction that the observed decrease in the frequency of cells expressing any one H-2^d-specific receptor in H-2^d mice would result under two conditions (Vance and Raulet, 1998). First, there must exist mechanisms to disfavor the coexpression of multiple self–class I–specific receptors by NK cells. This premise was supported by the finding that expression of a Ly49A transgene in all developing NK cells of H-2^d mice clearly

inhibits the expression of a second H-2^d-specific receptor, Ly49G2 (Held and Raulet, 1997). Second, H-2^d-specific receptor genes must be significantly more common than H-2^b-specific receptor genes in the germline receptor set (Vance and Raulet, 1998). The present data support the latter prediction.

The demonstration that class Ia tetramers bind to Ly49 receptors indicates that caution must be applied when using these reagents to detect specific T cell receptors, since the reagents may instead be detecting Ly49 receptors expressed on T cells (Ortaldo et al., 1998). The tetramer results have several implications concerning the nature of the Ly49–class I interaction. First, they go farther than previous studies in providing direct evidence for a physical interaction between class I molecules and Ly49 receptors. Second, since all tetramers except D^d were produced in bacteria and are therefore unglycosylated, the results provide compelling evidence that Ly49 binding to most class I molecules does not require glycosylation of the class I molecules. That class I–linked glycans might play a role in Ly49 binding had been suggested by the lectin-like structure of these receptors. Our results are in accord with previous studies demonstrating that mutagenesis of the acceptor sites for N-glycosylation in D^d did not prevent Ly49A binding (Lian et al., 1998; Matsumoto et al., 1998). The tetramer data do not, however, rule out the possibility that interaction of Ly49 receptors with class I-linked glycans might alter the affinity or specificity of the interaction.

The third significance of the tetramer binding studies concerns the role of MHC-bound peptides in the interaction. Previous results demonstrated that Ly49A does not discriminate D^d-bound peptides as tested in functional studies (Correa and Raulet, 1995; Orihuela et al., 1996). Along the same lines, the new data presented here indicate that Ly49C exhibits little, if any, capacity to discriminate peptides bound to K^d (Figure 6) or D^b (Figure 5; data not shown). Ly49I, however, reacted with only two of the five K^d–peptide complexes tested. The three complexes that did not bind to Ly49I bound well to Ly49C, demonstrating their integrity. These complexes showed essentially no reactivity with Ly49I-transfected cells, suggesting an appreciable reduction in the affinity compared to Ly49I binding to the other two K^d/peptide tetramers. The peptides that were compatible with Ly49 binding included a viral peptide (influenza HA^{204}) and a self-peptide (JAK1^{55}), indicating no general theme in the origin of active peptides. It remains to be determined whether the discrimination of peptides by Ly49I observed with tetramers also holds in functional tests and whether the peptide residues actually provide binding energy to the interaction or, alternatively, sterically impede the interaction.

A surprising finding was that D^b-tetramers efficiently stained Ly49C-transfected cells (Figure 5A). No appreciable binding of Ly49C to D^b could be detected in the cell–cell adhesion assay by antibody blocking or by employing cells from *K*$^{b-/-}$ mice that express D^b normally (Figures 3 and 4). Furthermore, previous functional (Yu et al., 1996) and binding (Brennan et al., 1996b) studies have failed to detect an interaction between Ly49C and D^b. D^b tetramers containing two distinct peptides stained Ly49C transfectants, arguing against the possibility that the failure to bind D^b on cells reflects a requirement for a specific peptide bound to D^b (Figure 5A; data not shown). The possibility must therefore be considered that the tetrameric D^b molecules produced in bacteria can in some cases exhibit different binding properties than D^b expressed in mammalian cells. One possibility is that the absence of glycans on the bacterially produced D^b reveals an epitope for Ly49C. Thus far, however, we have been unable to enhance binding of Ly49C transfectants to *K*$^{b-/-}$ cells by treating the latter cells with glycosidase (data not shown). It is also conceivable that the use of human β2m instead of mouse β2m in these tetramers could account for the unexpected binding behavior.

Another apparently anomalous finding in the tetramer studies was that D^d and K^b tetramers failed to bind well to Ly49I despite the clear evidence of binding observed in the cell–cell adhesion assay. Possibly relevant to these data is our observation that binding of D^d tetramers to Ly49A$^+$ NK cells appears to be highly sensitive to Ly49A surface expression levels. By mAb staining, the levels of Ly49A are reduced by 2- to 3-fold on NK cells from H-2^d mice compared to those from H-2^b mice. However, while the D^d tetramers are very effective in staining Ly49A$^+$ NK cells from H-2^b mice, they barely stain Ly49A$^+$ NK cells from congenic H-2^d mice (data not shown). Thus, tetramer binding appears to fall off sharply at a threshold Ly49 receptor density, consistent with the multivalent binding requirements expected of tetramers. Variations in affinity and in the levels of different Ly49 receptors on transfected cells could therefore explain why some class I–Ly49 interactions cannot be detected in our tetramer assays. Another possibility is that the peptides in the grooves of the D^d and K^b tetramers are not compatible with Ly49I binding. This explanation is plausible in light of the finding that Ly49I discriminates K^d tetramers complexed with different peptides.

An additional conclusion of our study was that the Ly49C proteins encoded by B6 and BALB strains, which differ in four amino acids, are indistinguishable in MHC specificity in the cell adhesion assay. Supporting this finding, Ly46C^{B6} and Ly49C^{BALB} also reacted equally with soluble MHC class I/peptide tetramers of the H-2^{b,d,k} haplotypes (data not shown). Based on functional studies, the possibility had been raised that these proteins differ in specificity (Yu et al., 1996), but it now appears that there must be another explanation for the functional results (George et al., 1997).

Finally, no interaction of Ly49D with D^d-expressing cells was detectable in our cell–cell adhesion studies, despite the functional evidence for this interaction. Furthermore, the D^d tetramers failed to detectably stain Ly49D-transfected COS-7 cells (data not shown). The data raise the possibility that the D^d–Ly49D interaction is weaker than some of the inhibitory interactions studied here. However, the possibility remains that the poor binding reflects a somewhat lower level of Ly49D on transfected cells than the levels of the inhibitory receptors, perhaps due to the requirement for association with cotransfected DAP12, or that Ly49D is more peptide or glycan specific than the inhibitory receptors. Ly49H-transfectants also failed to bind to class I molecules in either the cell–cell adhesion assay or the tetramer studies (data not shown). However, the significance of these

data is suspect, because the level of Ly49H on transfectants was substantially lower than the levels of the other Ly49 receptors studied (data not shown).

These results provide the most comprehensive picture to date of MHC binding by different Ly49 receptors and go a long way toward providing a basis for analyzing the overall repertoire of MHC specificities expressed by NK cells in a given mouse strain. A complete picture of the repertoire will require the incorporation of recently discovered inhibitory receptors, including the CD94/NKG2 receptors that react with a peptide derived from signal sequences of D region molecules that is presented by the Qa-1^b class Ib molecule (Vance et al., 1998). In addition, additional Ly49 genes have been discovered at the genomic level in B6 mice (McQueen et al., 1998); whether these receptors are functionally expressed remains to be determined. Once a complete panel is available, several other important questions can be addressed, such as whether NK cell self-tolerance can be accounted for by the expression of self-MHC-specific inhibitory receptors on each NK cell and whether congenic mice of different MHC haplotypes exhibit distinct inhibitory receptor repertoires.

Experimental Procedures

Animals and Generation of Transgenic Mice

C57BL/6J (H-2^b, B6), C57BL/10J (H-2^b, B10), B10.D2/nSnJ (H-2^d, B10.D2), B10.BR/SgSnJ (H-2^k, B10.BR), B10.M/Sn (H-2^f, B10.M), B10.Q/SgJ (H-2^q, B10.Q), B10.RIII(71NS)/Sn (H-2^r, B10.RIII), B10.S/SgMcdJ (H-2^s, B10.S), B10.SM(70NS)/Sn (H-2^v, B10.SM), B10.HTG/2Cy (H-2^g, B10.HTG), B10.D2(R107)/Eg (H-2^{i7}, B10.D2(R107)) and B10.A(2R)/SgSnJ (H-2^{h2}, B10.A(2R)) mice were purchased from the Jackson Laboratory. B6-$\beta 2m^{-/-}$ mice (Zijlstra et al., 1990) had been backcrossed five times to B6 mice before intercrossing. B10.BR-$\beta 2m^{-/-}$ and B10.D2-$\beta 2m^{-/-}$ mice were previously described (Held et al., 1996b), as were Ly49A transgenic mice (line 2) (Held et al., 1996a). $K^{b-/-}$, $D^{b-/-}$, and $K^{b-/-}D^{b-/-}$ mice of the B6 background have been described (Perarnau et al., 1999). For Ly49G2 transgenic mice, the complete coding sequence of the Ly49G2 cDNA (identical to the sequence published by Smith et al., 1994) was subcloned into the pHSE expression cassette (Pircher et al., 1989) and injected into fertilized (B6 × CBA/J F2) eggs. The line described here (line 5) was backcrossed at least four times to B6 mice and contained approximately eight transgene copies as determined by Southern blotting.

Cloning of Ly49 cDNAs

Ly49^{B6} cDNAs were cloned by RT–PCR from total B6 LAK cell or EL-4 RNA using Ly49 family member specific primers. Ly49A (amino acid sequence identical to that in Held et al., 1995), Ly49B (sequence from Wong et al., 1991), Ly49C (sequence from Stoneman et al., 1995), Ly49E, Ly49F and Ly49G2 (sequences from Smith et al., 1994), and Ly49I (identical to sequence by Brennan et al., 1996a) cDNAs were obtained. An HA-peptide epitope tag (amino acid sequence YPYDVPDYA) was added to the C termini of Ly49B, Ly49E, and Ly49A (the latter only for staining comparisons in Figure 1). The Ly49C^{BALB} cDNA (sequence from Wong et al., 1991) was cloned from a C.B-17 SCID LAK cell cDNA library (kindly provided by P. Mathew and V. Kumar). The cDNAs were subcloned into the pME18S expression vector.

Adhesion Assay

At day −1, COS-7 cells (CRL1651, purchased from ATCC) were seeded at 2 × 10^6 cells/T75 flask. At day 0 (50%–80% confluency), they were transfected with 5 μg of pME-Ly49 (cDNA) or pME-Ly49G-2^{inverse} as a control using lipofectamine (GIBCO–BRL) according to the manufacturer's instructions. At day 1, the transfectants were passaged into 6-well plates (Falcon). At day 0, RBC-depleted spleen cell suspensions were cultured at 2 × 10^6/ml with 2.5 μg/ml concanavalin A (Con A). At day 1, they were pulsed with 2.5 μCi/ml ^{3}H-thymidine (Amersham). At day 2, washed COS-7 transfectants were overlaid in 1.5 ml medium with Con A blasts at 1–5 × 10^6 cells/ml. After 2 hr incubation at 37°C, the wells were washed gently 5 times with prewarmed RPMI medium to remove unbound cells. The remaining conjugates were lifted from the plates using PBS/trypsin and transferred into 96-well plates. The plates were harvested automatically and the radioactivity was determined using a β-counter. Typically, approximately 1%–2% of the input radioactivity bound to mock transfectants; in the case of a strong binding interaction, 10%–20% of the input radioactivity bound to the corresponding Ly49-transfected COS-7 cells. The binding index was determined as cpm Ly49-cDNA transfected well/cpm Ly49G2^{inverse} transfected well. COS-7 transfectants were monitored for Ly49 surface expression by staining with anti-Ly49 mAbs after lifting the cells with PBS/0.02%EDTA.

MHC Class I Tetramers

The following tetramers were generated as described in bacteria in association with human β2m and were conjugated to PE or APC (Altman et al., 1996; Busch et al., 1998): K^b, SEV9 (Sendai virus); D^b, gp33 (LCMV); D^b, NP396 (LCMV); K^d, JAK1 55–63 (mouse); K^d, HA 204–212 (influenza virus); K^d, LLO 91–99 (*Listeria*); K^d, p60 217–225 (*Listeria*); K^d, mpl 84–92 (*Listeria*); L^d, NP118 (LCMV); D^k, MT389 (polyoma virus).

D^d tetramers were produced in insect cells, in association with mouse β2m. The murine β2m and D^d cDNAs were cloned into the dual expression transfer vector pAcUW31. The sequences encoding the leader and extracellular domains of D^d (ending at W274) were followed by sequences encoding a His tag and a BirA biotinylation sequence (LNDIFEAQKIEWH). Recombinant D^d was produced using a baculovirus expression system (Summers and Smith, 1987). pHIV peptide (RGPGRAFVTI, 0.25 mg/ml) was added to the supernatants of infected High 5 cells (Invitrogen) and stirred slowly for 12 hr at 4°C. Recombinant D^d was purified by Ni-NTA superflow chromatography (Qiagen) followed by gel filtration chromatography using a Superdex 200 FPLC column (Pharmacia). The integrity and specificity of all tetramers was confirmed by staining antigen-specific T cells or T cell lines.

Antibodies

The following mAbs were used: 4D11 (anti-Ly49G2 [Mason et al., 1995]), JR9–318 (anti-Ly49A [kindly provided by J. Roland]), A1 (anti-Ly49A [Nagasawa et al., 1987]), 4E5 (anti-Ly49D [Mason et al., 1996]), 14B11 (anti-Ly49C, Ly49F, Ly49H, Ly49I [Corral et al., 1999]), 34–5–8S (anti-D^{d}α1/α2 [ATCC HB-102]), 15–1–5P (anti-D^k, K^k [ATCC HB-53]), and Y3 (anti-K^b [ATCC HB-176]). Purified mAbs were conjugated to biotin or FITC. Anti-CD3-PE was purchased from Pharmingen, anti-HA from BAbCO, and donkey-anti-mouse Ig-PE (DaMIg-PE) from Jackson.

Staining and Flow Cytometry

Cell staining with antibodies was performed as described (Held et al., 1996b). For tetramer staining, the cells were preincubated for 20 min with 500 μg/ml streptavidin (Molecular Probes), washed, and stained for 1 hr with PE- or APC-conjugated tetramers. They were washed twice and fixed in PBS/1% formaldehyde before analysis.

Mixed Lymphocyte Reaction

Nylon wool–passed lymph node cells (>85% CD3$^+$) from H-2^b transgenic and nontransgenic mice were used as responder cells at 1 × 10^5 cells/round-bottom well with 5 × 10^5 irradiated (2500 rad) spleen cells as stimulators. Cultures were pulsed with 0.5 μCi ^{3}H-thymidine on day 3 and harvested on day 4. Stimulation indices (SI) were determined as cpm with allogeneic stimulator cell / cpm with H-2^b stimulator cells. From these values we determined the "inhibition index" as SI for nontransgenic responder cells / SI with transgenic responder cells.

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